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Response of heterogeneous ribonuclear proteins (hnRNP) to ionising radiation and their involvement in DNA damage repair

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Abstract

Purpose: To determine the relationship between heterogeneous nuclear ribonucleoproteins (hnRNP) and DNA repair, particularly in response to ionising radiation (IR).

Materials and methods: The literature was examined for papers related to the topics of hnRNP, IR and DNA repair.

Results: HnRNP orchestrate the processing of mRNA to which they are bound in response to IR. HnRNP A18, B1, C1/C2 and K interact with important proteins from DNA Damage Response (DDR) pathways, binding DNA-dependent protein kinase (DNA-PK), the Ku antigen (Ku) and tumour suppressor protein 53 (p53) respectively. Notably, irregularities in the expression of hnRNP A18, B1, K, P2 and L have been linked to cancer and radiosensitivity. Sixteen different hnRNP proteins have been reported to show either mRNA transcript or protein quantity changes following IR. Various protein modifications of hnRNP in response to IR have also been noted: hnRNP A18, C1/C2 and K are phosphorylated; hnRNP C1/C2 is a target of apoptotic proteases; and hnRNP K degradation is controlled by murine double minute ubiquitin ligase (MDM2). Evidence points to a role for hnRNP A1, A18, A2/B1, C1/C2, K and P2 in regulating double-stranded break (DSB) repair pathways by promoting either homologous recombination (HR) or non-homologous end rejoining (NHEJ) repair pathways following IR.

Conclusions: HnRNP proteins play a pivotal role in coordinating repair pathways following exposure to IR, through protein-protein interactions and transcript regulation of key repair and stress response mRNA. In particular, several hnRNP proteins are critical in coordinating the choice of HR or NHEJ to repair DSB caused by IR.

Keywords: *hnRNP, radiation, DNA DSB repair, homologous recombination, transcription factors*

Acronyms: 14-3-3σ, stratifin; ATM, Ataxia Telangiectasia Mutated kinase; ATR, Ataxia Telangiectasia Rad3 related kinase; CHiP, Chromatin immunoprecipitation; CIRP, cold-inducible RNA-binding protein; Cs-137, cesium-137; Co-60, cobalt-60; DDR, DNA damage response; DSB, double-strand breaks; DNA-PK, DNA-dependent protein kinase; Gy, gray; GSK3β, glycogen synthase kinase 3 beta; HDM2, human homologue of murine double minute; hnRNP, Heterogeneous ribonucleoprotein; HR, homologous recombination; IR, ionising radiation; Ku, Ku antigen, 70kDa and/or 80 kDa subunit; MDM2, murine double minute, a p53 ubiquin ligase (mouse); Mre11, meiotic recombination 11 homologue; Nbs1, Nirbrin; NGF, Nerve Growth Factor; NHEJ, non-homologous end joining; p21, Cyclin-dependent kinase inhibitor 1A; p53, tumour protein 53; PIKK, phosphatidylinositol 3-kinase-like kinase; Rad50, Rad50 homologue; RGG, arginine-glycine-glycine; RNP, ribonuclear proteins; RPA, Replication protein A; siRNA, short interfering RNA; TERT, Telomerase Reverse Transcriptase; TLS/FUS, Translocated/Fusion; hnRNP P2; UV, ultraviolet; UV-B, medium wavelength ultraviolet; UV-C, shortwave ultraviolet; UP1, Unwinding Protein 1; XRCC4, X-ray repair in Chinese hamster cells 4 protein.

Introduction

In eukaryotic cells pre-mRNA molecules are packaged, processed, exported and localised to production sites in the cytoplasm by a dynamic complex of diverse proteins known as ribonuclear proteins (RNP). These proteins follow RNA from transcription in the nucleus to translation in the

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cytoplasm (Krecic and Swanson 1999, Dreyfuss et al. 2002). RNP are involved in gene regulation through a plethora of protein-protein, protein-RNA and protein-DNA interactions that connect them to the major regulatory networks of the cell.

Most of the human genome is represented by primary transcripts, including non-coding portions (Weinstock 2007). Only 2–3% of this transcriptional output is RNA that codes for protein, while the non-coding majority is spliced from pre-mRNA and sent to the exosome for processing (Mattick 2003). Additionally, the majority of eukaryotic mRNA expresses distinct localisation patterns throughout the cell (e.g., 71% of 3370 genes analysed by florescent in situ hybridisation assays in embryonic *Drosophila* cells showed clear distribution patterns) (Lécuyer et al. 2007). These critical cellular regulatory functions, transcript processing and localisation are regulated by RNP complexes which escort pre-mRNA to the cytoplasm. The overwhelming diversity of these proteins, their multitude of structural conformations, binding specificities and isoforms, implies that RNA transport and processing are highly specialised functions (Krecic and Swanson 1999, Dreyfuss et al. 2002).

Heterogeneous nuclear ribonucleoproteins (hnRNP or sometimes hnrp) make up a significant subclass of known RNP. They are defined by three attributes: (1) They can be co-immunoprecipitated; (2) They show RNA binding capacity; and (3) They do not belong to other defined classes of RNP such as small nuclear RNP (Dreyfuss et al. 1993, Shyu and Wilkinson 2000). HnRNP were first described as a group of 6 chromatin associated RNA binding proteins which bound nascent polymerase II transcripts (Dreyfuss et al. 1993). Subsequent co-immunopurification, gene cloning and sequence analysis revealed a group of 30 abundant proteins and other less common hnRNP isoforms (Dreyfuss et al. 2002). The number of known hnRNP continues to grow and sometimes well studied proteins are later identified as hnRNP. For this reason, nomenclature inconsistencies exist in the literature. This review will defer to the 'hnRNP' designation and make note of alternative names where relevant.

HnRNP partially compose, bind or unbind to an evolving and unique complex of RNP and RNA which determines the fate of transcripts (Dreyfuss et al. 2002). They are well tied to the control networks of the cell through interactions with DNA and proteins. HnRNP do not necessarily exhibit homology to one another, but there is, very often, substantial overlap between their structures and functions.

This document will focus on the role of specific hnRNP in the cellular response to ionising radiation (IR). IR plays a role in both the induction and the treatment of cancers. Within the cell, exposure to IR may cause direct and indirect DNA damage, genotoxic stress, general transcription arrest and necessitate the initiation of repair pathways (Ward 1988). To begin the DNA damage response (DDR) pathway six phosphatidylinositol 3-kinase-like kinases (PIKK) are activated, including ataxia telangiectasia mutated (ATM) protein, ATM and rad3 related (ATR) protein, ATM nerve growth factor (NGF), and DNA-dependent protein kinase (DNA-PK). Activated, these proteins phosphorylate downstream target proteins which coordinate the process of DNA repair (Abraham 2004).

Double-stranded breaks (DSB) in genomic DNA are considered to be the most deleterious form of DNA damage caused by IR. DSB may be repaired through either the homologous recombination (HR) pathway regulated in part by Recombination protein A homologues (hRad51, hRad52 and hRad54) or through the non-homologous end joining (NHEJ) pathway regulated in part by DNA-PK, and Ku antigen (Ku) (see (Sonoda et al. 2006, Weinstock et al. 2006) for review). With respect to aiding cell survival and introducing mutations into genome, both of these repair pathways have their own peculiar strengths and weaknesses. While HR has the potential to restore the complete sequence information for damaged genes, too much HR may lead to a loss of genomic heterogeneity, gross chromosomal rearrangements and ultimately tumorgenesis (Henning and Stürsbecher 2003); also, HR is possible only during late S and G2 phases of the cell cycle after the cellular DNA has been replicated. On the other hand, NHEJ can occur at any point of the cell cycle but it is more error prone than HR and therefore less likely to restore the functionality of damaged sequences at the site of DSB; nevertheless, NHEJ generally prevents mitotic death and severe structural rearrangements to the genome. Ultimately, the cell must execute a delicate and complex balance between NHEJ, HR and the initiation of apoptosis to limit genomic damage, mutation burden and energy drain of the organism following radiation exposure.

This review will attempt to aggregate and summarise the current research to provide a basis for further lines of inquiry. There are many examples in the literature wherein hnRNP proteins have been shown to respond to stresses which may overlap with the effects of IR including ultraviolet (UV) irradiation and various DNA damaging chemicals like cisplatin or phleomycin. Exhaustive study of these citations is the topic for a different review and such data will be used only when they corroborate evidence of IR effects on hnRNP.

hnRNP A/B family of proteins

The hnRNP A/B family together constitutes 60% of the hnRNP present in a cell (Beyer et al. 1977).

The family includes multiple proteins and splice variants, most notably hnRNP A1, A2, B1 and B2. The hnRNP A/B family shares significant structural homology, each member has two RNP-motif RNA-binding domains and a variable glycine-rich auxiliary domain at the carboxyl terminus (Dreyfuss et al. 1993). All hnRNP A/B family proteins shuttle dynamically between the nucleus and cytoplasm (Pinol-Roma and Dreyfuss 1992). However, different proteins and splice variants have shown different expression profiles across cell types (Kamma et al. 1999) and have been implicated in a variety of tasks differentiated by protein and splice-variant.

hnRNP A1

HnRNP A1 protein and its splice variants comprise the most common gene products in the hnRNP A/B family (Beyer et al. 1977). HnRNP A1 regulates alternative splicing of RNA polymerase II transcripts in conjunction with the serine/arginine rich protein family (Mayeda and Krainer 1992, Caceres et al. 1994, Yang et al. 1994). Splice variant Unwinding Protein 1 (UP1) plays a critical role along with the Telomerase Reverse Transcriptase (TERT) and the Telomerase-Associated Protein 1 (TEP1) in forming the telomerase holoensyme responsible for telomere maintenance and promoting cell line immortalisation (LaBranche et al. 1998, Dallaire et al. 2000, Fiset and Chabot 2001, Lin et al. 2001). Several reports directly link changes in hnRNP A1 expression to the cellular response to IR (Khodarev et al. 2001, Lin et al. 2001, Jen and Cheung 2003), others indicate responses to other DNA damage inducing agents, UV (van der Houven van Oordt et al. 2000, Rundhaug et al. 2005) and cisplatin (Yim et al. 2006). Others suggest that hnRNP A1 is affected by more general cellular stresses such as hypoxia (Denko et al. 2000), heat shock (Guil et al. 2006) and osmotic stress (van der Houven van Oordt et al. 2000, Guil et al. 2006).

Messenger RNA for hnRNP A1 was identified by gene expression microarrays to be among 126 other radiation responsive transcripts, in a screen of pooled lymphoblast isolates from 10 individuals. Isolated cells were cesium-137 (Cs-137) γ-irradiated with 3 or 10 Gy (gray) ex vivo and RNA samples were collected at 0, 1, 2, 6, 12 and 24 h following irradiation. After 3 Gy exposure, cells' hnRNP A1 transcript expression peaked after 2 h with expression levels 2 times the basal amount followed by a rapid decline to basal levels by the 6th hour post-irradiation. One hour following 10 Gy irradiation, cells' hnRNP A1 mRNA expression increased two-fold and remained high through the remaining 23-h time period (Jen and Cheung 2003). Khodarev et al. analysed microarray data from U87 and HEL-C fibroblasts grown in culture treated with 0, 1, 3, or 10 Gy followed by 5 h of incubation. Results indicate that U87 cells show a consistent decline in hnRNP A1 transcript quantity, relative to controls, after 1, 3 and 10 Gy treatments with values of 0.99, 0.57 and 0.39, respectively. HEL-C fibroblasts demonstrated the opposite trend, increasing in transcript quantity with higher doses (1.65-fold after 1 Gy, 1.81-fold after 3 Gy, and 4.53-fold after 10 Gy exposure) (Khodarev et al. 2001).

hnRNP A1 splice variant UP1 and hTERT compose the telomerase holoensyme. It was found that telomerase activity is down-regulated over a 48-h period following 30 Gy exposure in Jukat and CEM-6, but not Raji cells (Lin et al. 2001); Lin et al. found that hTERT was upregulated in the same period both as mRNA by Real Time PCR (RT-PCR) and protein by Western Blot. However, these authors did not explore the effects of exposure on UP1 expression directly nor hypothesised a role for the protein in the subsequent down-regulation of telomerase activity (Lin et al. 2001).

Immunofluorescence studies have revealed cytoplasmic accumulation of hnRNP A1, induced following shortwave ultraviolet (UV-C) irradiation (180 J/m^2), in 3–15% of NIH-3T3, COS, 293 and HeLa cell lines detectable 2 h following treatment and peaking at 5 h (van der Houven van Oordt et al. 2000). Osmotic stress in the same study resulted in similar cytoplasmic accumulation of hnRNP A1 (van der Houven van Oordt et al. 2000). The authors showed that p38 kinase activation was both necessary and sufficient to cause cytoplasmic accumulation of hnRNP A1 (van der Houven van Oordt et al. 2000). Other studies have reported stress granule accumulation of hnRNP A1, observed by immunofluorescence, in NIH-3T3 cells in response to heat shock, a high osmolarity environment and oxidative stress (Guil et al. 2006).

Given the cumulative evidence gathered, the simplest explanation for the action of hnRNP A1 is that in response to IR it accumulates in the cytoplasm and stress granules. This migration could play a role in targeting and silencing of bound transcripts as well as optimising cellular resources for stress response activity. Depletion of nuclear levels of functional hnRNP A1 may contribute to a reduction of telomerase activity, progression through the cell cycle, and in some cell lines precede changes in hnRNP A1 production through unknown mechanisms in the subsequent hours or days. Given these conclusions, hnRNP A1 may be an important molecule to study to provide targeted treatment for infinitely proliferating cells and to further understand the IR response pathway.

hnRNP A2/B1

HnRNP A2 and hnRNP B1 are two closely related splice variants of the hnRNP A/B family which differ

by a 12 amino acid stretch present near the amino terminus of hnRNP A2 (Burd et al. 1989). Because of this close homology, the two are often treated together in the literature under the name hnRNP A2/B1. However, important distinctions between these variants have been shown. HnRNP A2 and hnRNP B1 show different expression profiles (Kamma et al. 1999). For example, hnRNP A2 is expressed at higher levels than hnRNP B1 in the adrenal gland and brain, while B1 is more prevalent in the intestines, heart and lung in adult rats (Kamma et al. 1999). Notably, hnRNP B1 expression levels are elevated in a variety of cancers including lung (Sueoka et al. 1999, Hamasaki et al. 2001, Sueoka et al. 2001), oral cavity (Goto et al. 1999) and esophagus (Matsuyama et al. 2000), and this overexpression can be used to detect the early stages of premalignant lesions (Goto et al. 1999, Sueoka et al. 2001).

Three studies have implicated hnRNP A2/B1 induction or relocalisation as an effect of irradiation or UV exposure. Gamble et al. used two-dimensional polyacrylamide gel electrophoresis to identify protein expression changes four hours following 0.5 Gy Co-60 γ-ray exposure of a human lung cell line. Among seven down-regulated proteins identified, they found hnRNP A2/B1 with a four-fold lower expression following treatment compared to controls (Gamble et al. 2000). Takao et al. performed microarray analysis of freshly cultured human keratinocytes 6 h following 100 J/m^2 medium wavelength (UV-B) irradiation. The hnRNP B1 mRNA transcript was identified among 88 other upregulated transcripts, and showed a 2.8-fold increase in transcript expression relative to controls (Takao et al. 2002). Cytoplasmic accumulation of hnRNP B1 was observed in 10–15% of green monkey kidney cells following UV exposure of 180 J/m^2 or osmotic shock as observed by immunofluorescence (van der Houven van Oordt et al. 2000).

It has also been reported that IR can lead to DNA-protein cross links with hnRNP A2/B1 (Barker et al. 2005). HnRNP A2/B1 was identified among 29 proteins in a DNAsol-Strip screen for DNA protein crosslinks following 1 Gy Co-60 γ-ray ionising irradiation under hypoxic conditions in Chinese Hamster Ovary cells (Barker et al. 2005). Interestingly, hnRNP A2/B1 has been proposed to inhibit DNA-PK following observations of co-immunoprecipitation of these proteins and in conjunction with a finding that rates of DSB repair increase following small interfering RNA (siRNA) knockdown of hnRNP A2/B1 in human bronchial epithelial cells (Iwanaga et al. 2005). Considering that DNA-PK is a major contributor to NHEJ, the effects of hnRNP B1 on the choice of repair mechanism, and therefore repair fidelity, may be significant (Figure 1a). The authors suggest that hnRNP A2/B1 may play a role in cancer promotion through the erroneous repair of DSB in hnRNP B1 upregulated tissues (Iwanaga et al. 2005).

Taken together, these results suggest an important and under-characterised role for hnRNP A2/B1 in radiation responses and cancer development specifically through the repair of DSB. Induction of hnRNP A2/B1 transcripts following radiation exposure shows some cell-type specificity and will require more exhaustive work on both A2 and B1 splice variants to resolve the question of the promotion or inhibition of pathways involved. While evidence reported by Iwanaga et al. that hnRNP B1 induction may lead to the erroneous repair of DSB offers a tantalising proposition, this line of research is still understudied, especially as it applies to the radiation response pathway.

A18 hnRNP

A18 hnRNP is a stress inducible protein, which was first cloned by low ratio hybridisation subtraction of rapidly induced transcripts in UV-irradiated Chinese hamster ovary cells (Fornace et al. 1988) and then in human cell lines (Sheikh et al. 1997). A protein with 90% amino acid homology was identified as a cold-inducible RNA-binding protein (CIRP) in mice (Nishiyama et al. 1997). A18 hnRNP has one conserved RNA binding domain and several repeats of an arginine-glycine-glycine (RGG) box – a single-stranded nucleic acid binding motif (Kiledjian and Dreyfuss 1992).

A18 hnRNP has been shown to bind selectively to the 3′ untranslated region of numerous mRNA sequences (Yang and Carrier 2001). Of 46 mRNA transcripts identified to bind selectively to A18 hnRNP, about 40% are known to be stress or UV responsive (Yang and Carrier 2001). An RKO cell line transfected with an antisense vector to reduce hnRNP A18 expression becomes UV sensitive (Yang and Carrier 2001). Another major group of A18 hnRNP binding mRNA are those coding for ribosomal proteins, which also play a role in stress responses (Sheikh and Fornace 1999). Interestingly, one of the transcripts found to associate with A18 hnRNP via its 3′ untranslated region is replication protein A (RPA), which plays major direct and indirect roles in DNA replication and repair, most importantly in DSB repair through HR (Fanning et al. 2006). This finding suggests a regulatory role for A18 hnRNP in the DDR pathway (Figure 2a). Moreover, RPA and A18 hnRNP are involved in the phosphorylation of tumour protein 53 (p53) (Ljungman 2007), linking p53 with the transcriptional stress response.

In response to UV exposure, A18 hnRNP protein is induced, peaking 4 h after treatment (Fornace et al. 1988, Yang and Carrier 2001). After

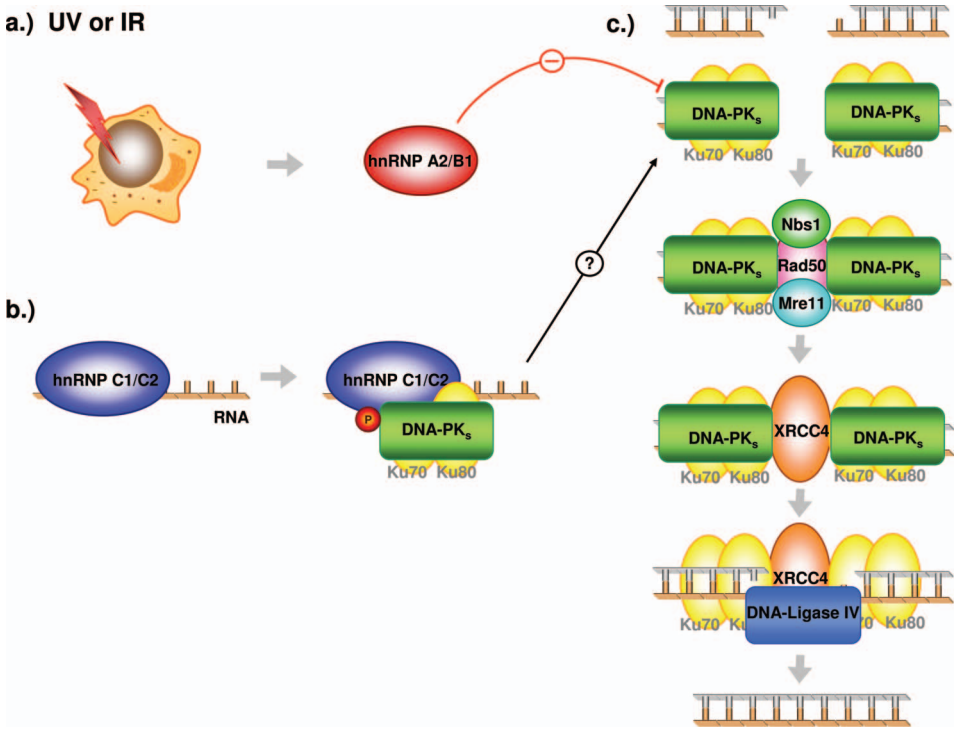

Figure 1. Non-homologous end joining repair (NHEJ). NHEJ repairs DSB through an error prone mechanism available at any point during the cell cycle. As opposed to HR, NHEJ is less likely to lead to gross chromosomal rearrangements. (a) UV and IR are known to induce hnRNP A2/B1 protein which has been proposed to inhibit DNA-PK (Iwanaga et al. 2005); (b) hnRNP C is phosphorylated by a complex of DNA-PK and Ku proteins when it is bound to RNA transcript; the effect on NHEJ is unknown (Shang et al. 2004, Lee et al. 2005); (c) The canonical NHEJ pathway is shown repairing a DSB beginning with DNA-PK and Ku protein binding, followed by Rad50 homologue (Rad50), Nirbrin (Nbs1), and meiotic recombination 11 homologue (Mre11) recruitment, displaced by X-ray repair in Chinese hamster cells 4 protein (XRCC4) which recruits DNA-Ligase IV culminating in the rejoining of the broken DNA strands (Weller et al. 2004, Sonoda et al. 2006, Weinstock et al. 2006).

UV-induction, the protein binds to and stabilises stress-inducible mRNA transcripts (Yang and Carrier 2001) and translocates them from the nucleus to the cytosol (Yang and Carrier 2001). UV treatment leads to A18 hnRNP phosphorylation of the RGG domain by glycogen synthase kinase 3 beta (GSK3β) (Kim and Kimmel 2000), which increases its transcript binding efficiency by two-fold (Yang et al. 2006). It is suggested that this phosphorylation may also play a role in cytoplasmic relocation of A18 hnRNP because of a similar relocation pathway discovered for protein nuclear factor of activated T-cells (NFATc) (Beals et al. 1997).

In a microarray analysis of human MOLT4 cells, induction of A18 hnRNP was found immediately after 5 Gy Cs-137 γ-irradiation and lasted 8 h (Barenco et al. 2006). In addition, one of transcripts known to selectively bind to A18 hnRNP is thioredoxin, which is known to be induced by X-ray irradiation, sources of oxidative stress and UV irradiation (Funasaka and Ichihashi 1997). Thioredoxin has been shown to be upregulated in some tumours and been associated with UV resistance (Yokomiso et al. 1995, Berggren et al. 1996, Grogan et al. 2000).

A18 hnRNP protein seems to be involved in coordinating the translocation of stress-response transcripts to the cytoplasm during the recovery period following exposure to a cellular stress. The response is general, apparently encompassing stresses ranging from IR to heat-shock (Fornace et al. 1988). At the same time, protein RPA, regulated by A18 hnRNP is involved in HR DNA repair.

Other miscellaneous hnRNP A/B family results

An hnRNP A/B type transcript (accession number NM_010448) was identified by microarray among 69 down-regulated transcripts in isolated bone marrow cells from C57BL mice 6 h after a whole body Co-60 γ-irradiation with 6.5 Gy. This transcript was 3-fold lower in irradiated than in control mice (Dai et al. 2006).

Another hnRNP A/B type transcript (accession number M65028) was identified by microarray among 126 other radiation responsive transcripts, in a screen of pooled lymphoblasts from 10 individuals (Jen and Cheung 2003). Isolated cells were Cs-137 γ-irradiated with 3 or 10 Gy and RNA was isolated at 0, 1, 2, 6, 12 and 24 h following

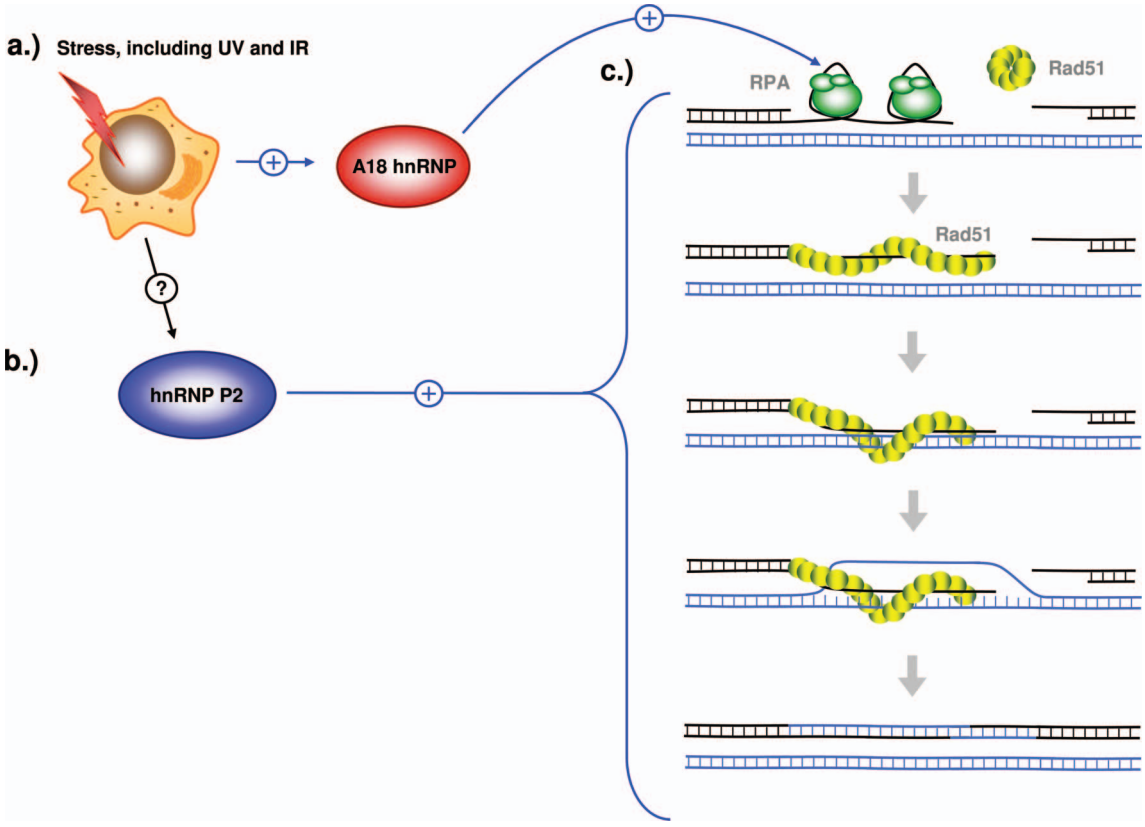

Figure 2. Homologous recombination (HR). HR repairs DSB using the broken strand's sister chromatid as a template. As opposed to NHEJ, HR may restore the complete sequence information for damaged genes. However, an excess of HR may lead to gross chromosomal rearrangements. (a) Many forms of stress including UV and IR are known to induce A18 hnRNP which exports RPA transcripts to the cytoplasm (Yang and Carrier 2001). Cytoplasmic localisation likely induces RPA which would facilitate HR; (b) Kuroda's group has found evidence that hnRNP P2 promotes homologous repair in mice through an unknown mechanism. The authors suggest that hnRNP P2 may provide an RNA template for HR (Kuroda et al. 2000); (c) A simplified view of the HR pathway details the roles of Rad51 and RPA in preparing the damaged DNA for restoration using the sister chromatid as a template (San Filippo et al. 2008).

exposure. Protein hnRNP A/B type M65028 mRNA showed a slow decline in expression from 2 h following either 3 or 10 Gy Cs-137 γ-irradiation and continuing throughout the 24-h time period at the end of which its expression was 2-fold lower than control levels.

HnRNP A3 was identified among 29 proteins in a DNAsol-Strip screen for DNA protein crosslinks following 1 Gy ionising irradiation under hypoxic or aerated conditions in Chinese Hamster Ovary cells (Barker et al. 2005).

HnRNP C1/C2

HnRNP C1 and hnRNP C2 are splice variants which differ by a 13 amino acid stretch present in the middle of the coding sequence of the C2 gene (Burd et al. 1989). Because of their significant homology, they are frequently referred to collectively as hnRNP C1/C2 or simply hnRNP C in the literature. HnRNP C1/C2 has been shown to play a role in mRNA transcript packaging, splicing, nuclear retention and mRNA stability (Krecic and Swanson 1999). Under normal conditions, it is located in the nucleoplasm, but not nucleoli (Lee et al. 2005). Messenger RNA and protein for hnRNP C1/C2 have been shown to respond to IR with transcript induction, chromatin-binding, chromatin-crosslinking and protein cleavage, described in studies detailed below. Because of these responses, it has been suggested that hnRNP C1/C2 plays a role in coordinating the DNA-damage response and radiation induced apoptosis pathways. However, no follow-up studies have been performed to elucidate the mechanisms of their action or the downstream effects that are dependent upon hnRNP C1/C2 responses to IR.

HnRNP C1/C2 can be isolated in screens for both DNA protein crosslinks and chromatin-binding proteins following IR. DNA crosslinking was observed 1 h following treatment with moderate doses of Co-60 γ-IR (1–4 Gy) in Chinese hamster ovary cells (CHO AA8) and human fibroblast cells (GM00637) especially under hypoxic conditions (Barker et al. 2005). Chromatin-binding was observed in HeLa cells 3 h after treatment with high doses of IR (25 Gy) (Lee et al. 2005). Such binding

was found in nuclei extracts from treated cells and was reversible with 0.5 M but not 0.35 M NaCl elution (Lee et al. 2005). These binding and cross-linking responses imply close proximity between hnRNP C1/C2 and chromatin. However, the localisation pattern of hnRNP C1/C2 does not change after exposure to ionising irradiation (van der Houven van Oordt et al. 2000, Lee et al. 2005), therefore hnRNP C1/C2 does not appear to bind specifically to damage sites. The authors suggest that hnRNP C1/C2 may play a 'global' role in orchestrating DNA repair pathways. It is possible that hnRNP C1/C2 coordinates changes in gene expression required for DNA repair pathways after irradiation through direct interaction with genomic DNA, DNA associated proteins and/or mRNA transcripts, but further study is required to validate this hypothesis.

A different report has indicated that hnRNP C1/C2 undergoes cleavage as the result of activation of the apoptotic proteases at 8 h post-treatment by high doses of IR (20 Gy) in Burkitt's lymphoma cells, BL30A (Waterhouse et al. 1996). Importantly, this response was not observed in a radiation resistant subline, BL30K, but could be induced in BL30K by a variety of apoptotic agents (etoposide, C8 ceramide, tetrandrine). The cleavage seems to be one result of a more general apoptosis pathway (Waterhouse et al. 1996). No follow-up studies were done to elucidate further the mechanisms or downstream effects of this cleavage.

Two microarray studies have indicated a change in expression levels of hnRNP C1/C2 transcripts following IR treatment. One study reported steadily decreasing transcript levels of hnRNP C1/C2 in the 24-h period following 3 Gy, but not 10 Gy Cs-137 γ-irradiation treatment of 10 different human lymphoblastoid cell lines (Jen and Cheung 2003). Another study found a $5\times$ decrease in transcript expression relative to control levels 6 h after 6.5 Gy Co-60 γ-irradiation treatment of bone marrow cells extracted from C57BL mice as compared to a sham-irradiated control (Dai et al. 2006). While these results imply a change in the transcription of hnRNP C1/C2 following radiation exposure, once again no follow-up studies were performed to connect these changes in expression levels to downstream effects on cells.

Experiments examining general stress response pathways have implicated a role for hnRNP C1/C2 in DNA damage repair mechanisms. One study showed that hnRNP C1/C2 binds to Ku protein if it is at the same time binding RNA transcripts and can be phosphorylated by the catalytic subunit of the DNA-PK complex (Figure 1b) (Shang et al. 2004). This suggests a possible role for hnRNP C1/C2 in DNA DSB repair through the non-homologous end-joining pathway (Lee et al. 2005). Other studies have connected hnRNP C1/C2 with telomere repair and maintenance. It has been shown using UV cross-linking and co-immunoprecipitation assays that hnRNP C1/C2 associates with the 6 bp U-tract of the RNA component of the human telomerase holoensymes in lung adenocarcinoma cells (H1299) (Ford et al. 2000). Telomerase activity assays on VA13 cells revealed that the association of hnRNP C1/C2 with telomerase correlates with its ability to access the telomere (Ford et al. 2000).

It is intriguing to speculate that the previously mentioned responses of hnRNP C1/C2 to IR are responsible for regulation of DDR pathways induced by IR and general maintenance of genomic stability. It may be that during stress-induced transcriptional arrest hnRNP C1/C2 lacks bound RNA and therefore stops interacting with Ku and DNA-PK, perhaps leading to changes in NHEJ activity. Further study is required to prove these hypotheses.

HnRNP K

HnRNP K has been implicated in the processes of chromatin remodeling and mRNA transcription, splicing, export and translation (Bomsstyk et al. 1997). It shuttles between the nucleus and cytoplasm and contains three RNA-binding domains which preferentially bind C-rich nucleic acids and protein interactive domain (Klimek-Tomcsak et al. 2004). This section will focus on work performed by the Moumen lab showing that hnRNP K acts as a transcriptional co-regulator of p53 targets following irradiation (Figure 3).

While p53's role in cell-cycle checkpoint arrest and the DDR pathway was identified 15 years ago by Lane (1992) and Efeyan and Serrano (2007), the work of the Moumen group on hnRNP K was not carried out until 2003. It is not surprising that an important regulatory protein like p53 has a corresponding transcript regulating hnRNP partner. The extensive work done by the Moumen group to elucidate the function of hnRNP K in this role has provided an example for future lines of research and added a new critical molecule to incorporate into models of DNA damage repair and other cellular responses to IR.

Proteomic analysis first identified hnRNP K among upregulated proteins in GM 14680 cells treated with 20 Gy of γ-IR. Subsequent immunoblot experiments revealed that hnRNP K protein levels increased 2- to 3-fold 15 min after treatment with IR or the radiomimetic drug phleomycin (Moumen et al. 2005). These protein levels returned to control level after 3 h in repair-proficient cells, but not in a line of cells deficient in DSB repair (180BR) in which hnRNP K expression remained elevated 12 h following irradiation (Moumen et al. 2005).

Through siRNA knockdown experiments in SAOS2 cells Moumen et al. found that hnRNP K

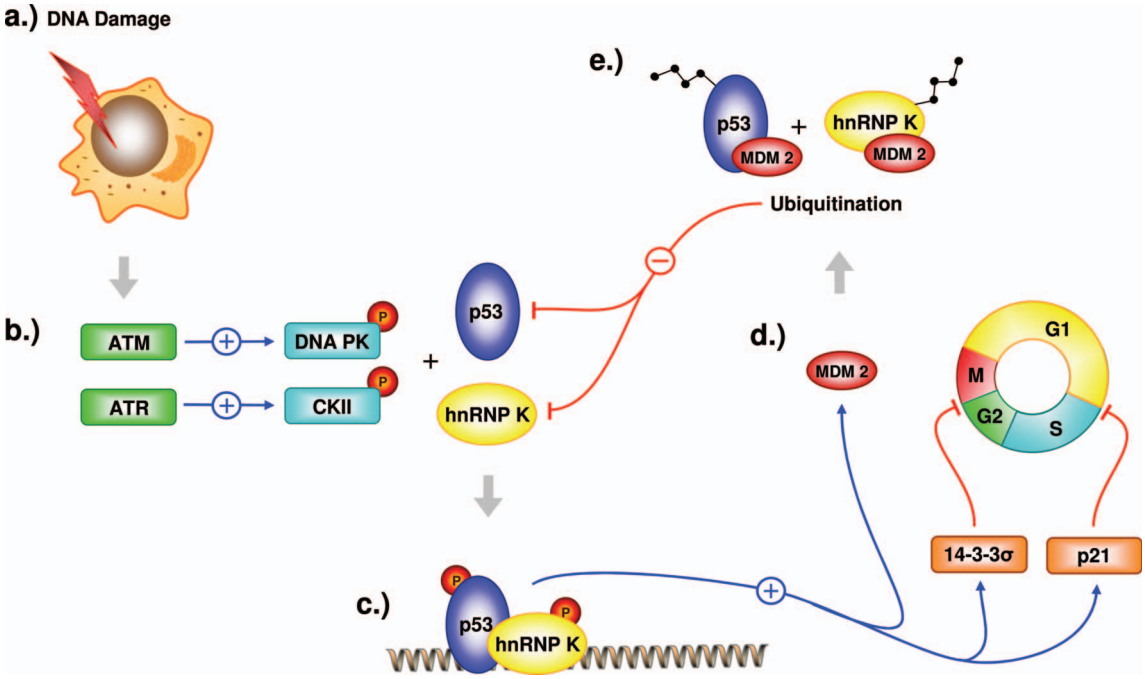

Figure 3. hnRNP K and p53 in the DNA damage response (DDR). The Moumen Lab showed that hnRNP K and p53 act as co-regulators. This figure presents a simplified view of the role of p53 in the DNA damage response pathway (Bullock and Fersht 2001) alongside hnRNP K functions discovered by the Moumen lab (Moumen et al. 2005). (a) Following DNA damage due to IR or other insult; (b) Proteins ATM and ATR induce DNA PK and CKII which phosphorylate p53 and hnRNP K; (c) Phosphorylated hnRNP K and p53 proteins bind co-dependently to p53 promoter sites resulting in transcript upregulation for a number of DDR genes; (d) P53/hnRNP K targets, p21 and 14-3-3σ halt cell cycle progression at G1 and G2 phases, respectively; (e) Another p53/hnRNP K target, murine double minute ubiquitin ligase (MDM2) plays an inhibitory role by adding ubiquitin to p53 and hnRNP K resulting in their degradation.

protein, like p53, is induced following irradiation due to cessation of the human homologue of murine double minute (HDM2) regulated ubiquitin based proteasomal degradation (Moumen et al. 2005). They went on to show, through further chemical inhibition and siRNA knockdown experiments that, like p53, hnRNP K induction was dependent on the action of ATM and ATR kinases (Figure 3b). ATM and ATR kinases are key regulatory proteins in the DDR pathway and function to phosphorylate and inhibit degradation of DDR proteins following IR (Abraham 2004, Shiloh et al. 2004).

To test for the specificity of observed hnRNP K induction, the group measured the effects of UV, heat shock, hypotonic and hypertonic treatments on hnRNP K protein expression in multiple cell lines. Of these, only UV (25 J/m^2 measured 1 h after treatment) leads to hnRNP K upregulation (Moumen et al. 2005). The authors suggest that the induction pattern of hnRNP K indicates that it is upregulated specifically in response to DNA lesions (Moumen et al. 2005)

Interestingly, Chromatin Immunoprecipitation (CHiP) assays indicated that hnRNP K and p53 are co-recruited to p53 responsive promoters and that hnRNP K acts to regulate mRNA targets of p53 controlled genes (Figure 3c) (Moumen et al. 2005). Cells treated with siRNA targeting hnRNP K prevented p53 recruitment to cyclin-dependent kinase inhibitor 1A (p21) and HDM2 promoter regions despite the fact that p53 stabilisation was not affected (Moumen et al. 2005). Furthermore, RT-PCR analysis of the U2OS cell line assessing expression of p53 transcript targets: p21, HDM2, 14-3-3σ (stratifin) and luciferase, confirmed that hnRNP K knockdown inhibited induction of these mRNA transcripts following irradiation (Figure 3d) (Moumen et al. 2005).

Because p53 plays a critical role in cell cycle arrest following an IR event, the authors went on to explore the role that hnRNP K might play in facilitating this p53-dependent cell cycle arrest. After hnRNP K knockdown by siRNA, γ-irradiated MRC5 fibroblast cells did not show their natural G1/S phase arrest (Moumen et al. 2005). Similarly, U2OS cells which are normally arrested at the G2/M phase following irradiation did not undergo cycle arrest when hnRNP K or p53 siRNA treatment was applied (Moumen et al. 2005). However, p53-deficient SAOS2 cells exhibited their characteristic G2/M phase arrest following irradiation regardless of hnRNP K knock-down (Moumen et al. 2005). These results suggest that hnRNP K is an essential cofactor of p53-dependent cell cycle checkpoints following IR.

From the Moumen lab's report, it is clear that hnRNP K is in part responsible for the fate of p53 regulated transcripts following irradiation. HnRNP K is one of downstream targets of ATR/ATM phosphorylation and HDM2 mediated degradation pathways (Figure 3b, 3e). In this, hnRNP K mirrors the behaviour of p53 and ultimately acts as a co-factor coordinating the downstream response of DDR pathways. However, the mechanisms and regulatory steps occurring that deliver hnRNP K-target transcripts to translation sites in the cytoplasm have yet to be elucidated. Transcripts may or may not be escorted directly by hnRNP K to the cytoplasm. Irrespective of that, these transcripts are almost certainly interacting with a variety of other regulatory proteins and RNA complexes before their ultimate translation.

Given the relationship between hnRNP K, p53 and the transcript targets of p53, it is not surprising that hnRNP K overexpression has been linked to multiple forms of cancer (Pino et al. 2003). Both yet-to-be-discovered transcript shuttling pathways and hnRNP K itself are prospective targets for anticancer therapies.

hnRNP P2

Protein hnRNP P2 more commonly known as translocation/fusion protein (TLS/FUS) (Calvio et al. 1995) contains an RNA binding motif flanked by RGG repeats (Delattre et al. 1992, Crosat et al. 1993, Rabbitts et al. 1993). In some human sarcomas and leukemias, the C-terminus of this protein is missing and the remaining amino acid sequence is fused to sequences coding unrelated transcription factors (Kuroda et al. 2000). These fusion products are invariably associated with myxoid and round cell liposarcomas (Crosat et al. 1993, Rabbitts et al. 1993).

Transgenic hnRNP $P2^{-/-}$ mice studied by Kuroda et al. display increased radiosensitivity, profound defects in spermatogenesis, and a mild defect in somatic growth as compared to wild type mice (Kuroda et al. 2000). In a survival study 18/20 adult transgenic mice died within 20 days of receiving whole body Co-60 γ-rays irradiation with 7 Gy, whereas control mice survived in 21/26 cases. Additionally, primary mouse embryonic fibroblasts isolated from hnRNP $P2^{-/-}$ mice showed consistently lower survival rates than wildtype isolated in the seven days following 3.5, 7, or 10.5 Gy irradiation (Kuroda et al. 2000).

The authors have proposed that hnRNP P2 may play a role in homologous DNA pairing and recombination, key aspects of HR repair (Figure 2b). With the evidence gathered thus far, there can be little doubt that hnRNP P2 plays a role in maintaining genomic stability, especially following genotoxic stresses. The cancer associated fusion proteins are likely to play a critical role in facilitating structural changes to the genome necessary for the development of malignancy. However, more work is needed to elucidate the mechanistic underpinnings of the role of hnRNP P2 in genomic maintenance.

Miscellaneous results

During the course of research a number of references were collected that showed a link between hnRNP transcript induction and IR, but did not fit with a greater body of evidence. These sources have been collected and summarised in (Table I). It is important to note that these citations are biased towards positive results and the majority of micro-array or proteomic studies involving IR treatment in the literature do not report changes to hnRNP transcripts or proteins. For this reason it may be assumed that the results accumulated are relevant to the particular treatment conditions of each experiment and not necessarily generalisable. Even with the above qualifications, there are some interesting conclusions to be drawn from the accumulated gene expression results. Some hnRNP are more represented than others when associated with gene expression changes following IR. Namely hnRNP E gene family members were each differentially regulated in three IR studies; hnRNP H, R and U were each noted in two studies; and hnRNP D, L and M were noted in only one study each. These results should guide the course of mechanistic research towards promising candidate hnRNP that are involved in DDR following irradiation.

Discussion

From the evidence accumulated, it is clear that hnRNP are critical regulatory proteins in the cellular response to IR and other stresses. It is also clear from the diversity of responses that have been noted, that biology is only beginning to characterise the role of these multi-faceted proteins in DDR regulation. Advances in molecular biology techniques, especially siRNA and florescence assays, will prove to be critical in furthering our understanding.

The most commonly observed effect of IR on hnRNP was transcript or protein induction. Sixteen hnRNP, A1, A2/B1, A18, AB Type (NM_010448), AB Type (M65028), C1/C2, D, E1, E2, H1, H3, K, L, M, R and U were noted in various studies as being IR inducible mRNA. Most of the observed effects appear to be cell type or growth phase specific and it is difficult to draw broad conclusions from the data. Rather, the induction effects thus far noted may serve as fruitful jumping off points for future research,

Table I. Miscellaneous microarray associations between hnRNPs and radiation exposure.

hnRNP/s	Observations	Src.
hnRNP E1 (PCBP1)	Microarray analysis identified hnRNP E1 among 128 down-regulated transcripts in the 24 h following 3 Gy or 10 Gy irradiation of pooled lymphoblast isolates from 10 individuals.	(Jen and Cheung 2003)
hnRNP E2 (PCBP2)	Microarray analysis identified hnRNP E2 among 69 down-regulated transcripts (0.22 fold of control) in RNA extracts of pooled bone marrow from 5 Male C57BL mice, 6 h following whole body irradiation with a single dose of 6.5 Gy.	(Dai et al. 2006)
hnRNP E2	Microarray analysis identified hnRNP E2 among 114 upregulated transcripts in growth arrested HEL-C fibroblasts and among 161 down-regulated transcripts in malignantly derived U87 fibroblasts 5 h following 1, 3, or 10 Gy irradiation.	(Khodarev et al. 2001)
hnRNP H1	Microarray analysis identified hnRNP H1 among 43 upregulated transcripts 1 h following 6 Gy irradiation of A172 cells. However, these results were not observed at other time points (0.5 or 6 h) or in other human glioblastoma cell lines U87MG and U13MG.	(Otomo et al. 2004)
hnRNP L	Microarray analysis identified hnRNP L among four down-regulated transcripts in time-dependent and independent analysis of bone marrow isolates collected from nine patients diagnosed with acute lymphoblastic leukemia who had received preventative cranial irradiation and subsequently developed secondary brain tumours.	(Edick et al. 2005)
hnRNP R	Microarray analysis found hnRNP R among 56 differentially regulated transcripts 72 h following 4 Gy irradiation of radiation-resistant HepG2 cells when compared to radiation-sensitive Hep3B cells which showed three-fold higher expression. However, the effect was not observed at other time points: 0, 3, and 24 h.	(Jeong et al. 2006)
hnRNPs D, H3, M, R, and U	Microarrays revealed gradual down regulation in hnRNPs D, H3, M, R, and U transcripts among 319 others in the 24 hours following 3 Gy, but not 10 Gy, ionising irradiation of lymphoblastoid cells.	(Jen and Cheung 2003)
hnRNP U	Microarray analysis identified hnRNP U among 59 down-regulated transcripts 3 h following 5 Gy X-ray irradiation of cultured human keratinocyte cells (58% of untreated expression).	(Koike et al. 2005)

especially in the cases of hnRNP E, H, R and U which have each been noted in multiple induction studies following IR, but lack follow-up research to describe the relevant DDR pathways they might connect to.

While hnRNP are characterised primarily as RNA transcript processing agents, there is a surprising lack of evidence of hnRNP relocalisation to the cytoplasm or stress granules following IR treatment. HnRNP A1 and B1 relocalisation has been noted in response to a variety of stresses, including UV, but not to IR treatment specifically (van der Houven van Oordt et al. 2000, Guil et al. 2006). It is likely that the dearth of evidence directly linking IR treatment and hnRNP relocalisation is due to a lack of investigation or the technical difficulties associated with IR studies. Further investigations into this response are likely to be fruitful, especially in the examples of hnRNP A1 and B1.

The most important evidence to emerge from the literature reviewed in this paper demonstrates that hnRNP function as upstream and co-dependent regulators of critical DDR proteins. Based on the data found in the literature thus far, it is possible to hypothesise that hnRNP optimise the cell's DDR response by regulating proteins which participate in HR, NHEJ and general transcriptional arrest.

HnRNP K has been shown to act as a critical co-factor of p53, necessary for p53-dependent cell cycle checkpoints and p53-dependent transcript induction following IR treatment. Further, like p53, hnRNP K is a downstream target of ATR/ATM phosphorylation and HDM2 mediated degradation which co-ordinates its rapid induction following irradiation (Moumen et al. 2005). Also, stress induced cytoplasmic relocalisation of hnRNP A1 corresponds to a reduction in telomerase activity (of which hnRNP A1 is a constituent) which contributes to cell cycle arrest following IR (Khodarev et al. 2001). Connection between hnRNP C1/C2 and telomerase activity were also noted and may be involved in cell cycle arrest.

Other hnRNP have been closely associated with HR and NHEJ DSB repair pathways. UV and IR have been shown to produce positive or negative induction of hnRNP B1 (Gamble et al. 2000, Takao et al. 2002). Upregulation of hnRNP B1 leads to DNA-PK inhibition (Iwanaga et al. 2005) and would favour the HR pathway, while down-regulation of hnRNP B1 would favour NHEJ. Many general stresses have been shown to upregulate A18 hnRNP, including UV and IR treatment, which in turn upregulates RPA favouring HR repair (Fornace et al. 1988, Yang and Carrier 2001, Barenco et al. 2006, Fanning et al. 2006). The interaction of

hnRNP C1/C2 with Ku and DNA-PK is dependent on an interaction between hnRNP C1/C2 and mRNA (Shang et al. 2004), which are generally down-regulated in response to stress because of transcriptional arrest. The dissociation of hnRNP C1/C2 with Ku and DNA-PK almost certainly has implications in the rate of NHEJ, which DNA-PK and Ku regulate, however it is difficult to determine if this would favour or oppose NHEJ without further evidence. Lastly, hnRNP P2 has been shown to be directly involved in HR repair and hnRNP $P2^{-/-}$ mice are radiosensitive (Calvio et al. 1995).

Conclusions

In light of the above associations between hnRNP and critical components of DDR, it is not surprising that mutations in a number of these proteins, hnRNP A18, B1, K, P2 and L, have been linked to cancer and radiosensitivity (Goto et al. 1999, Sueoka et al. 1999, 2001, Kuroda et al. 2000, Matsuyama et al. 2000, Hamasaki et al. 2001, Yang and Carrier 2001, Pino et al. 2003, Edick et al. 2005). It seems that hnRNP proteins are integral in determining the delicate balance of mutation prone and high fidelity repair processes, prevention of genomic rearrangements, orchestration of transcript mobilisation post IR stress, and cooperation with transcription factors such as p53. For these reasons, the continued study and elucidation of hnRNP roles holds the promise of better understanding the mechanisms which promote cancer and ultimately may lead to useful therapeutic treatments.

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